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Gastrointestinal Cancer

External Quality Assessment for *KRAS* Testing Is Needed: Setup of a European Program and Report of the First Joined Regional Quality Assessment Rounds

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Learning Objectives

After completing this course, the reader will be able to:

1. Identify the most frequent errors made in *KRAS* testing in this study and the possible consequences for a patient.
2. Describe factors that could increase the chance of an error during *KRAS* testing.

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ABSTRACT

The use of epidermal growth factor receptor–targeting antibodies in metastatic colorectal cancer has been restricted to patients with wild-type *KRAS* tumors by the European Medicines Agency since 2008, based on data showing a lack of efficacy and potential harm in patients with mutant *KRAS* tumors. In an effort to ensure optimal, uniform, and reliable community-based *KRAS* testing throughout Europe, a *KRAS* external quality assessment (EQA) scheme was set up. The first large assessment round included 59 laboratories from eight different European countries. For each country, one regional scheme organizer prepared and distributed the samples for the participants of their own country. The samples included unstained sections of 10 invasive colorectal carcinomas with known *KRAS* mutation status. The samples were centrally validated by one of two reference laboratories. The laboratories were allowed to use their own preferred method for histological evaluation, DNA isolation, and mutation analysis. In this study, we analyze the setup of the *KRAS* scheme. We analyzed the advantages and disadvantages of the regional scheme organization by analyzing the outcome of genotyping results, analysis of tumor percentage, and written reports. We conclude that only 70% of laboratories correctly identified the *KRAS* mutational status in all samples. Both the false-positive and false-negative results observed negatively affect patient care. Reports of the *KRAS* test results often lacked essential information. We aim to further expand this program to more laboratories to provide a robust estimate of the quality of *KRAS* testing in Europe, and provide the basis for remedial measures and harmonization. *The Oncologist* 2011;16:467–478

INTRODUCTION

Epidermal growth factor receptor (EGFR) targeting therapies have been developed for the treatment of patients with metastatic colorectal cancer. Initially, these therapies were given to unselected populations, but novel insights suggested that these therapies would be effective only in wild-type *KRAS* populations [1]. Mutations in the *KRAS* gene are found in 30%–40% of colorectal tumors and are accompanied by a poor response to cetuximab or panitumumab [2–5]. Independent reanalysis of eight randomized clinical trials showed a lack of efficacy for these therapies when a *KRAS* codon 12 or codon 13 mutation was present [6]. Based on these results, the recommended use of these drugs was amended by both the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), with important differences, however. The FDA issued a recommendation in 2009 against the use of these drugs in patients with tumors mutated in codon 12 or codon 13 of *KRAS*, but a label change for the drugs will require two additional elements. First, a single assay for the detection of the seven codon 12 and codon 13 mutations needs to be validated, fulfilling class III premarket approval requirements. Second, all randomized clinical trials need to be reassessed uniformly with this assay, with consistent results on the negative predictive impact, before a label change, including the in vitro diagnostics, will be declared. These data are expected in 2011. In Europe, the EMA took a different approach, changing the approval of these drugs for use in wild-type *KRAS* populations only, based on the retrospective datasets. This has important implications because the exact mutations to be tested are not specified nor is the methodology. Consequently, community testing for *KRAS* was developed in 2008, with many laboratories using different techniques and sometimes testing different mutation panels. Because the correctness of the *KRAS* test result is of utmost importance for good patient care, we set up a quality control scheme aimed at (a) assessing the performance of *KRAS* testing in Europe, (b) providing remedial measures if necessary, and (c) ensuring uniform performance of the laboratories over time by repeat testing rounds [7, 8]. Mutations in the *KRAS* gene can be detected by several different molecular methods [9, 10]. There is no gold standard methodology. Every test may have limitations that must be carefully considered before clinical implementation. Theoretically, the method of choice could vary according to the characteristics of the tumor sample (tumor content and DNA quality) as well as the mutations being searched for [11]. In the U.S., there has been a choice of setting the TheraScreen®: K-RAS Mutation Kit (CE-IVD) (Qiagen Manchester, Manchester, U.K.), an allele-specific quantitative polymerase chain reaction (QPCR) assay detecting seven mutations on codon 12 and codon 13, as the gold standard with which other tests may be compared in the future. However, this method detects only the most common activating mutations, limiting the possibilities of evaluating the effects of the less common ones. Performance characteristics and test limitations are established and documented through the process of test validation. External quality assessment (EQA) is one of the essential steps in the validation of clinical tests [12]. All this has implications for a quality control scheme assessing the proficiency of different test methods.

A *KRAS* EQA scheme was set up in eight different European countries. The purpose of this scheme is to monitor

the quality of *KRAS* testing and improve the quality by providing a means for evaluation and education of laboratories. The *KRAS* EQA scheme evaluated the performance of *KRAS* testing, including the correct identification of *KRAS* mutations, percentage tumor cells, and reporting of test results. This program was organized by a European quality assurance (QA) working group, supported by the European Society of Pathology (ESP) and in close collaboration with existing regional and national QA programs. The development of an EQA scheme provides a unique opportunity to document variations in *KRAS* diagnostic testing and to evaluate the need for such a scheme. This study describes the initial development of a regional *KRAS* EQA scheme in 2009 in different European countries and aims to provide a baseline picture of the accuracy and reliability of the analysis of the *KRAS* test to identify areas of particular difficulty in testing procedures and to assess the feasibility of a European EQA scheme.

METHODS

Scheme Organization

Coordination Center

The coordination of the regional *KRAS* EQA scheme in 2009 was supported by the ESP QA working group, the department of Pathology of the Radboud University Nijmegen Medical Centre (medical and technical expert), and the Biomedical Quality Assurance Research unit of the University of Leuven (scheme coordinator). The existing national EQA programs for *KRAS* were contacted to stimulate closer cooperation in the organization of *KRAS* EQA schemes.

Regional Laboratories

Laboratories from eight European countries (Austria, Belgium, Denmark, France, Greece, Spain, Sweden, and The Netherlands) participated in the regional *KRAS* EQA scheme. In each of these countries, one regional scheme organizer was responsible for the preparation, validation, and shipment of the samples to the different laboratories in their country. The regional scheme organizers of this *KRAS* EQA scheme were selected based on four criteria: (a) good experience with *KRAS* testing on a routine basis, (b) access to samples (blocks) that could be used for the EQA program (conforming to the national legal requirements of the use of patient samples), (c) ability to organize and execute national and regional EQA rounds in collaboration with the *KRAS* EQA coordination center (including preparation and sending of samples), and successful participation in a previous pilot EQA scheme that was running in May to June 2009 (Dequeker et al., submitted manuscript). The regional scheme organizers were free to choose the samples for the *KRAS* EQA scheme with the following conditions: (a) There was a consensus in the number of wild-type versus mutated samples that had to be included in the scheme. (b) It was not necessary to have all kind of mutations, but having as many as possible different mutations was suggested. (c) A sample from either the primary tumor or a metastasis could be used. (d) The samples had to contain four to six slides of 4–5 μm or three slides of 10 μm or a combination. (e) Given the importance of performing high-quality tests, the first slide and the last slide of each sample needed to have at least 30% tumor cells and these slides need to be archived. (f) It was the responsibility of the regional scheme provider to collect the 10 samples and to foresee enough material from each sample for all the laboratories who were participating in the EQA scheme.

The regional scheme organizers validated the samples before shipping them to participants.

Reference Laboratory

The regional scheme organizers sent one sample set to the central reference laboratory in Nijmegen. In the Dutch scheme, the central reference laboratory of Nijmegen took the role of scheme organizer. Therefore, in this scheme, the laboratory in Leuven was the reference laboratory. The central reference laboratory in Nijmegen used dideoxysequencing as the genotyping method and the reference laboratory in Leuven used the TheraScreen® DxS kit. In every scheme, the method of the central reference laboratory was different from the method of the scheme organizer. Thus, results were always verified by at least two different methods. The role of the reference laboratory was to perform a second validation of the samples. The result of this second validation was compared with the results of the validation of the regional scheme organizers. If these results were different, the samples were retested with both methods and, if necessary, with other methods as well, for confirmation of the genotype.

Participants

Through the newsletter and the website of the ESP, laboratories were invited to participate in the EQA scheme. There were no criteria set for participating. In total, 59 laboratories (including the regional laboratories) from eight different European countries participated in the regional *KRAS* EQA scheme in 2009.

Samples

Each regional scheme organizer sent three to six consecutive unstained sections from 10 tissue blocks to each participating laboratory in their country (Fig. 1). The samples

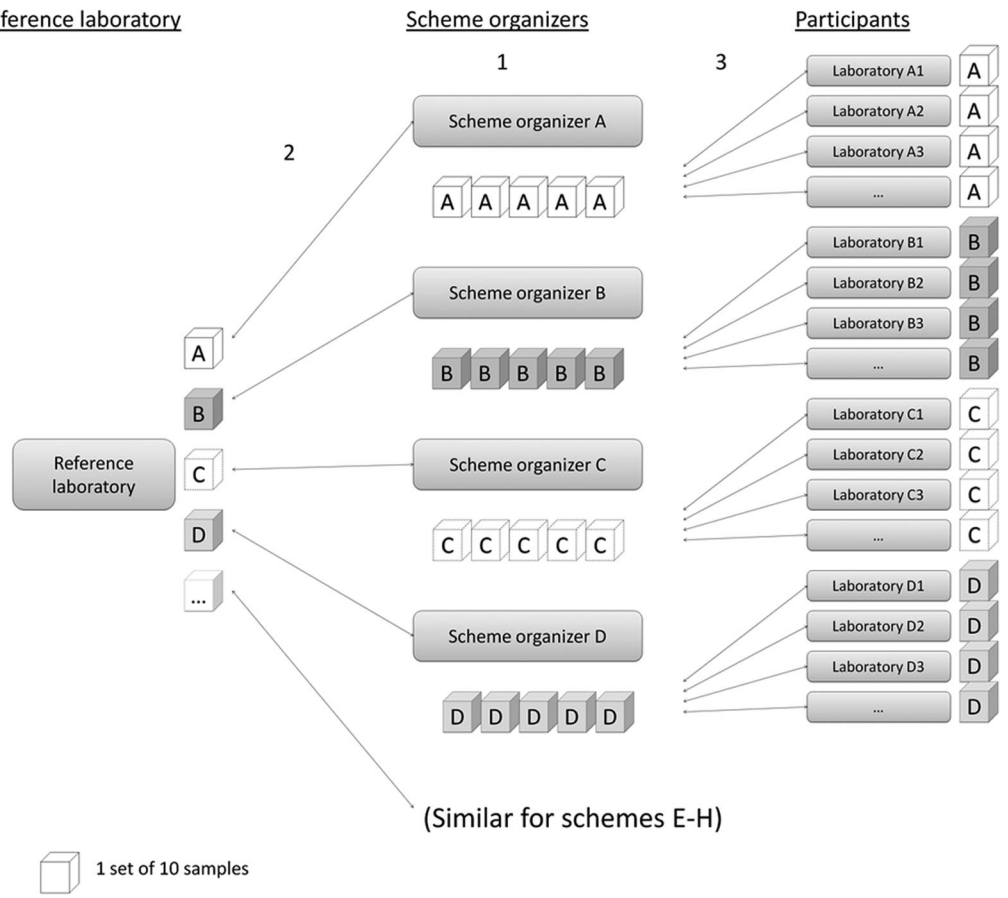

Figure 1. Schematic representation of the preparation and distribution of the samples in the *KRAS* external quality assessment scheme. (1) represents the first validation of the samples by the scheme organizer before sending the samples. (2) represents the second, central validation of the samples by the reference laboratory. (3) represents the analysis of the samples by the participating laboratories.

contained formalin-fixed, paraffin-embedded tissue from invasive colorectal carcinomas. The regional scheme organizers were asked to select eight samples with and two samples without a *KRAS* mutation. The sections were clearly labeled with a sample identification number and serial section number. Slides were divided such that laboratories did not always receive the higher or lower sections of the block. The last sections that were cut were sent to the reference laboratory. All mutations were located in codon 12 and codon 13. The different mutations present in this EQA scheme were c.37G>C (p.G13D), c.34G>C (p.G12D), c.34G>T (p.G12C), c.35G>T (p.G12V), c.34G>C (p.G12R), c.34G>A (p.G12S), and c.35G>C (p.G12A), comprising the most frequent alterations detected in tumors from patients with colorectal cancer [13]. The two most frequent mutations in routine diagnostic analysis, that is, c.34G>C (p.G12D) (36.0%) and c.35G>T (p.G12V) (21.8%), were present in all regional schemes. All laboratories were asked to stain the first section with hematoxylin and eosin (H&E) for histological evaluation using their routine protocol. The other sections could be used for DNA isolation and *KRAS* mutation detection.

Data Analysis

Results had to be submitted to the regional scheme organizer within 10 working days after receiving the samples. In addition, participants had to fill in general questions about the laboratory and *KRAS* testing.

We used a numerical scoring system. One point was awarded when the genotype was scored correctly or a mutation was missed that was not included in their standard assay. No points were awarded when the genotype was scored incorrectly. In cases of a double mutation, one point was awarded when at least one of the two mutations was found. So, in total, a maximum of 10 points (100%) could be obtained when all 10 samples were correctly genotyped. To exclude differences in genotyping resulting from the sampling of the tumor material, we analyzed if the laboratories that received the sections before and after this sample made the same mistake. The submitted results and the raw data

were analyzed by the *KRAS* coordination center in Leuven, together with the regional scheme organizers and experts at the reference laboratory.

Scoring of the Reports

Each participant was asked to send written reports of the first three samples, like the laboratories would normally do on a routine basis. The scoring results of the reports were used for educational purposes only and were not taken into account for successful participation in the EQA scheme. Analysis of the reports was based on the International Organization for Standardization (ISO) 15189:2007 requirements for medical laboratories and a guideline document developed by the College of American Pathologists regarding reporting of molecular results [14]. We applied these guidelines for *KRAS* test reports and defined 17 items that should be included in a good report for *KRAS* testing (Table 1). Each item was awarded one point if present; in some cases, we awarded 0.5 points (e.g., if the report had a title but it was not clearly distinguished from the rest of the report).

The mutation nomenclature for *KRAS* mutations had to comply with the guidelines from the Human Genome Variation Society (HGVS, http://www.hgvs.org) to stimulate uniform and unequivocal description of sequence changes. These state that nucleotide number 1 should correspond to the A of the ATG translation initiation codon. The description of all variants is preceded by a letter indicating the type of reference sequence used; "c." relates to a coding DNA sequence and "p." related to a protein sequence (Table 2).

Table 1. Different items used for scoring of reports of the *KRAS* external quality assessment scheme

Item	Description
1	Sampling/arrival date
2	Sample number
3	Date of report
4	Signature
5	Unique identifier on each page • For example, by lab identifier, name . . .
6	Total pages • Page 1 of 2, 1/2 (not 1,2,3, . . .)
7	Name/address referral person
8	Nature of the sample • Paraffin section, biopsies, formalin-fixed paraffin-embedded section, . . .
9	Percentage of tumor cells
10	Reason for testing • *KRAS* testing, presence of *KRAS* mutation, . . .
11	Genotype
12	Use of correct nomenclature
13	Interpretation of the data • Comments/results and conclusion/ discussion
14	List of mutations tested
15	Method used • Name of commercial kit or noncommercial assay • Version number (if applicable)
16	Report title • Refers to *KRAS* testing or *KRAS* molecular diagnosis and clearly distinguished from the rest of the report
17	Refers to therapy • Positive test for *KRAS* indicates low response to anti–epidermal growth factor receptor therapy (cetuximab, panitumumab)

According to the International Organization for Standardization (ISO) 15189:2007 standard and Gulley et al. (2007) [14].

RESULTS

First and Second Validation

The first analysis of the samples was carried out by the regional scheme organizers before sending the samples. The validation of the samples was carried out by the reference laboratory. The results of the scheme organizer and reference laboratory were compared (Table 3). When different results were obtained, the sample was retested.

The test results of sample KRASA09.04 showed discrepant values. To analyze the difference in genotype results, the sample was retested by the reference laboratory by different methodologies. The reference laboratory detected the mutation c.35G>T (p.G12V) with a standard PCR and dideoxysequencing. Subsequent coamplification at lower denaturation temperature PCR and cloning of the PCR products revealed a mixture of wild-type, c.35G>T (p.G12V), and c.34_35delinsCT (p.G12L) alleles. Because this last mutation was not detected by standard PCR, it was less present in the sample analyzed in the reference laboratory. Therefore, the G>C alteration on c.34 is considered a second hit on the c.35G>T allele. Both genotyping results (c.34G>C (p.G12R) and c.35G>T (p.G12V)) were considered correct. Reanalyzing the raw data of this sample by the laboratory of the regional scheme organizer of subscheme A with the TheraScreen® DxS kit showed the same result (a high signal for a T at c.35 and a very low signal for a C at c.34).

Analysis of Results by Participants

Results of the participants were compared with the result of the reference laboratory (Table 3). In subscheme D and sub-

Table 2. Frequency of mutations in the regional *KRAS* EQA scheme

Genotype in EQA sample	Number indicating the frequency that the mutation was analyzed in subscheme								Total in *KRAS* EQA scheme	Frequency in routine diagnosis in (%)[a]
	KRAS A	*KRAS B*	*KRAS C*	*KRAS D*	*KRAS E*	*KRAS F*	*KRAS G*	*KRAS H*		
c.35G>A (p.G12D)	18	7	12	20	7	14	14	7	99 (16.8%)	36.0
c.35G>T (p.G12V)	18	7	6	5	14	7	28	14	99 (16.8%)	21.8
c.38G>A (p.G13D)	18	7	9	5	14	7	14	14	88 (14.9%)	18.8
c.34G>T (p.G12C)	9	7		10	14	7	14	7	68 (11.5%)	8.0
c.34G>A (p.G12S)		7				7	28	7	49 (8.3%)	6.5
c.35G>C (p.G12A)		14			7	7	14		42 (7.1%)	6.0
c.34G>C (p.G12R)		7				7		7	21 (3.6%)	1.3
c.34_35delinsCT (p.G12V and p.G12R)	9								9 (1.5%)	0.1
Wild-type	18	14	3	10	14	14	28	14	115 (19.5%)	

[a]Results from Neumann et al. (2009) [13].
Abbreviation: EQA, external quality assessment.

scheme H, all results of participants were identical to the results of the first and second validation. In subscheme A, there were four laboratories (including the reference laboratory) unable to interpret the results of KRASA09.03 because of poor DNA quality in the sample. In two other subschemes (B and E), technical failures or isolation failures were present. Discrepant values were seen in the analysis of sample KRASA09.04, which was reanalyzed as a double mutation by the reference laboratory and the laboratory of the scheme organizer. Of 10 participating laboratories (including the reference laboratory), three reported a c.34G>C (p.G12R) mutation, four report a c.35G>T (p.G12V) mutation, one found no mutation, and two laboratories could not analyze the sample because of technical reasons. The other mistakes were distributed over the different mutations. In five subschemes (A, B, C, F, and G), there were laboratories with false-positive or false-negative results (Table 3). In only subscheme C was the same mistake made by multiple laboratories (KRASC09.08 tested twice as wild type). In none of the other subschemes were recurrent mistakes made in one of the samples. In three subschemes (B, E, and G), two mutations were detected, of which one was correct. In only one laboratory was a wrong mutation detected. In total, 41 laboratories reported all 10 genotypes correctly (100%) when compared with the correct result, 14 laboratories made one genotype mistake (90%), and four laboratories made two mistakes (80%). The average genotyping score for all laboratories that participated in the regional *KRAS* EQA scheme in 2009 was 95%. Not every subscheme contained the same mutations. In subscheme C, only three different mutations were present, whereas in subscheme B and subscheme F seven different mutations were present (Table 2). There was no difference in mistakes between frequent mutations and less frequent mutations. Participants were also asked to select the appropriate region of the section for DNA isolation and to estimate the percentage of tumor cells in this sample. There was very high variation in the determination of the percentage tumor cells. In several samples, there were high differences between the values, for example, labs that detected 10%–20% of tumor cells while others detected 90%–100% (a difference of 80%) (Fig. 2). No relation was seen between the level of the section and the percentage of tumor cells estimated, indicating that observer variability was the main cause of the large variation.

Description of Study Population

Together with submission of the results, participants were asked to fill in a list with general questions about the laboratory and *KRAS* testing. All 59 laboratories that participated provided this information. All these laboratories perform *KRAS* mutation testing for clinical purposes. Of the 59 participating laboratories, 19 laboratories were accredited according to ISO 15189 or ISO 17025. Another five laboratories were certified according to ISO 9001. There was no statistically significant difference in genotyping accuracy between ISO-accredited and nonaccredited laboratories. In 2009, the number of *KRAS* tests performed in these laboratories was in the range of 30–1,200 (according to the statements in the questionnaire given by the partici-

Table 3. Genotype results of the different subschemes

	KRASA09.01	KRASA09.02	KRASA09.03	KRASA09.04	KRASA09.05	KRASA09.06	KRASA09.07	KRASA09.08	KRASA09.09	KRASA09.10
Reference laboratory	c.34G>T (p.G12C)	Wild-type	Technical failure[5]	c.35G>T (p.G12V)[4,5]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
Scheme organizer A	c.34G>T (p.G12C)	Wild-type	c.35G>A (p.G12D)[5]	c.34G>C (p.G12R)[4,5]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
Consensus result	c.34G>T (p.G12C)	Wild-type	c.35G>A (p.G12D)	c.35G>T (p.G12V) + c.34G>C (p.G12R)[4]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
1–3	c.34G>T (p.G12C)	Wild-type	c.35G>A (p.G12D)	c.35G>T (p.G12V)[4]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
4	c.34G>T (p.G12C)	Wild-type	Technical failure	Technical failure	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
5	c.34G>T (p.G12C)	Wild-type	Technical failure	c.34G>C (p.G12R)[4]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	c.35G>A (p.G12D)[1]	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
6	c.34G>T (p.G12C)	Wild-type	c.35G>A (p.G12D)	c.34G>C (p.G12R)[4]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
7	c.34G>T (p.G12C)	Wild-type	Technical failure	c.35G>T (p.G12V)[4]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
8	c.34G>T (p.G12C)	Wild-type	c.35G>A (p.G12D)	Wild-type[1]	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.35G>A (p.G12D)
Slide details	1 slide of 4 μm and 2 slides of 10 μm									
	KRASB09.01	**KRASB09.02**	**KRASB09.03**	**KRASB09.04**	**KRASB09.05**	**KRASB09.06**	**KRASB09.07**	**KRASB09.08**	**KRASB09.09**	**KRASB09.10**
Reference laboratory	c.38G>A (p.G13D)	c.35G>C (p.G12A)	c.35G>C (p.G12A)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.34G>C (p.G12R)	c.35G>T (p.G12V)	Wild-type	c.34G>A (p.G12S)
Scheme organizer B	c.38G>A (p.G13D)	c.35G>C (p.G12A)	c.35G>C (p.G12A)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.34G>C (p.G12R)	c.35G>T (p.G12V)	Wild-type	c.34G>A (p.G12S)
1–2	c.38G>A (p.G13D)	c.35G>C (p.G12A)	c.35G>C (p.G12A)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.34G>C (p.G12R)	c.35G>T (p.G12V)	Wild-type	c.34G>A (p.G12S)
3	c.38G>A (p.G13D)	c.35G>C (p.G12A) + c.34G>A (p.G12S)[3]	c.35G>C (p.G12A) + c.34G>A (p.G12S)[3]	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.34G>C (p.G12R)	c.35G>T (p.G12V)	Wild-type	c.34G>A (p.G12S)
4	c.38G>A (p.G13D)	c.35G>C (p.G12A)	c.35G>C (p.G12A)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.34G>C (p.G12R)	Wild-type[1]	Wild-type	c.34G>A (p.G12S)
5	c.38G>A (p.G13D)	c.35G>C (p.G12A)	c.35G>C (p.G12A)	c.35G>C (p.G12A) + c.35G>A (p.G12D)[3]	Wild-type	c.34G>T (p.G12C)	c.34G>C (p.G12R)	c.35G>T (p.G12V)	Wild-type	c.34G>A (p.G12S)
6	c.38G>A (p.G13D)	c.35G>C (p.G12A)	c.35G>C (p.G12A)	c.35G>A (p.G12D)	Technical failure	c.34G>T (p.G12C)	c.34G>C (p.G12R)	c.35G>T (p.G12V)	Wild-type	c.34G>A (p.G12S)
Slide details	6 slides of 4 μm									
	KRASC09.01	**KRASC09.02**	**KRASC09.03**	**KRASC09.04**	**KRASC09.05**	**KRASC09.06**	**KRASC09.07**	**KRASC09.08**	**KRASC09.09**	**KRASC09.10**
Reference laboratory	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.38G>A (p.G13D)	Wild-type	c.35G>T (p.G12V)
Scheme organizer C	c.35G>A (p.G12D)	Wild-type[1]	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.38G>A (p.G13D)	Wild-type	c.35G>T (p.G12V)
Consensus result	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	Wild-type	Wild-type	c.35G>T (p.G12V)
1	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	Wild-type[1]	Wild-type	c.35G>T (p.G12V)
2	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	c.38G>A (p.G13D)	c.35G>A (p.G12D)	Wild-type[1]	Wild-type	c.35G>T (p.G12V)
Slide details	5 slides of 5 μm									
	KRASD09.01	**KRASD09.02**	**KRASD09.03**	**KRASD09.04**	**KRASD09.05**	**KRASD09.06**	**KRASD09.07**	**KRASD09.08**	**KRASD09.09**	**KRASD09.10**
Reference laboratory	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	Wild-type	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.34G>T (p.G12C)	c.35G>A (p.G12D)
Scheme organizer D	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	Wild-type	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.34G>T (p.G12C)	c.35G>A (p.G12D)
1–4	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.35G>A (p.G12D)	c.35G>T (p.G12V)	Wild-type	c.35G>A (p.G12D)	c.35G>A (p.G12D)	c.34G>T (p.G12C)	c.35G>A (p.G12D)
Slide details	1 slide of 3 μm and 2 slides of 6 μm									

(continued)

Table 3. (Continued)

	KRASE09.01	KRASE09.02	KRASE09.03	KRASE09.04	KRASE09.05	KRASE09.06	KRASE09.07	KRASE09.08	KRASE09.09	KRASE09.10
Reference laboratory	Wild-type	c.35G>T (p.G12V)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>C (p.G12A)	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>T (p.G12V)
Scheme organizer E	Wild-type	c.35G>T (p.G12V)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>C (p.G12A)	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>T (p.G12V)
1–4	Wild-type	c.35G>T (p.G12V)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>C (p.G12A)	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>T (p.G12V)
5	Wild-type	Isolation failure	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>C (p.G12A)	Isolation failure	c.34G>T (p.G12C)	c.35G>T (p.G12V)
6	Wild-type	c.35G>T (p.G12V)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>C (p.G12A) and c.34G>A (p.G12S)[3]	c.38G>A (p.G13D)	c.34G>T (p.G12C)	c.35G>T (p.G12V)
Slide details	6 slides of 4 μm									
	KRASF09.01	**KRASF09.02**	**KRASF09.03**	**KRASF09.04**	**KRASF09.05**	**KRASF09.06**	**KRASF09.07**	**KRASF09.08**	**KRASF09.09**	**KRASF09.10**
Reference laboratory	c.35G>A (p.G12D)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.34G>A (p.G12S)	c.35G>C (p.G12A)	c.35G>A (p.G12D)
Scheme organizer F	c.35G>A (p.G12D)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.34G>A (p.G12S)	c.35G>C (p.G12A)	c.35G>A (p.G12D)
1–4	c.35G>A (p.G12D)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.34G>A (p.G12S)	c.35G>C (p.G12A)	c.35G>A (p.G12D)
5	c.35G>A (p.G12D)	c.35G>T (p.G12V)[2]	Wild-type	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Wild-type	c.34G>T (p.G12C)	c.34G>A (p.G12S)	c.35G>C (p.G12A)	c.35G>A (p.G12D)
6	c.35G>A (p.G12D)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.38G>A (p.G13D)	Suspect c.35G>A (p.G12D)[1]	c.34G>T (p.G12C)	c.34G>A (p.G12S)	c.35G>C (p.G12A)	c.35G>A (p.G12D)
Slide details	1 slide of 4 μm and 2 slides of 10 μm									
	KRASG09.01	**KRASG09.02**	**KRASG09.03**	**KRASG09.04**	**KRASG09.05**	**KRASG09.06**	**KRASG09.07**	**KRASG09.08**	**KRASG09.09**	**KRASG09.10**
Reference laboratory	c.34G>A (p.G12S)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>T (p.G12C)	Wild-type	c.35G>C (p.G12A)	c.35G>T (p.G12V)	c.34G>A (p.G12S)
Scheme organizer G	c.34G>A (p.G12S)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>T (p.G12C)	Wild-type	c.35G>C (p.G12A)	c.35G>T (p.G12V)	c.34G>A (p.G12S)
1–10	c.34G>A (p.G12S)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>T (p.G12C)	Wild-type	c.35G>C (p.G12A)	c.35G>T (p.G12V)	c.34G>A (p.G12S)
11	c.34G>A (p.G12S)	c.35G>A (p.G12D)	c.35G>T (p.G12V)[1]	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>T (p.G12C)	Wild-type	c.35G>C (p.G12A)	c.35G>T (p.G12V)	c.34G>A (p.G12S)
12	c.34G>A (p.G12S)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V)	Wild-type[1]	Wild-type	c.35G>C (p.G12A)	c.35G>T (p.G12V)	c.34G>A (p.G12S)
13	c.34G>A (p.G12S)	c.35G>A (p.G12D)	Wild-type	c.38G>A (p.G13D)	c.35G>T (p.G12V) and c.38G>A (p.G13D)[3]	c.34G>T (p.G12C)	Wild-type	c.35G>C (p.G12A)	c.35G>T (p.G12V)	c.34G>A (p.G12S)
Slide details	3 slides of 10 μm									
	KRASH09.01	**KRASH09.02**	**KRASH09.03**	**KRASH09.04**	**KRASH09.05**	**KRASH09.06**	**KRASH09.07**	**KRASH09.08**	**KRASH09.09**	**KRASH09.10**
Reference laboratory	c.38G>A (p.G13D)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.34G>A (p.G12S)
Scheme organizer H	c.38G>A (p.G13D)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.34G>A (p.G12S)
1–6	c.38G>A (p.G13D)	c.35G>A (p.G12D)	Wild-type	c.34G>T (p.G12C)	c.38G>A (p.G13D)	c.35G>T (p.G12V)	c.34G>C (p.G12R)	Wild-type	c.35G>T (p.G12V)	c.34G>A (p.G12S)
Slide details	5 slides of 4 μm									

Thickness and number of samples sent in the scheme are indicated in the table.
1–4: Result of laboratories 1 to 4 (have the same result).
Consensus result: Result after reanalysis by the reference laboratory and regional organizer.
[1]Wild-type instead of mutation or mutation instead of wild-type.
[2]Mutation found, but wrong mutation.
[3]Two mutations found, of which one is correct.
[4]One of two mutations found of sample with double mutation.
[5]Discrepancy between the results of the regional organizer and the reference laboratory.

pants). There was a lot of variation in the number of samples analyzed per year in each laboratory. The majority analyzed 0–250 samples per year (73%). Thirty percent of the laboratories performed >250 tests per year. Only one laboratory performed >1,000 tests per year.

The two most frequently used *KRAS* mutation detection methods were the commercial TheraScreen® DxS (48%) and dideoxysequencing (28%). Mistakes were made using both commercial kits and in-house validated methods. The results obtained for the (double) mutation in sample KRASA09.04 depended on the method used. All laboratories that detected the c.35G>T (p.G12V) mutation were us-

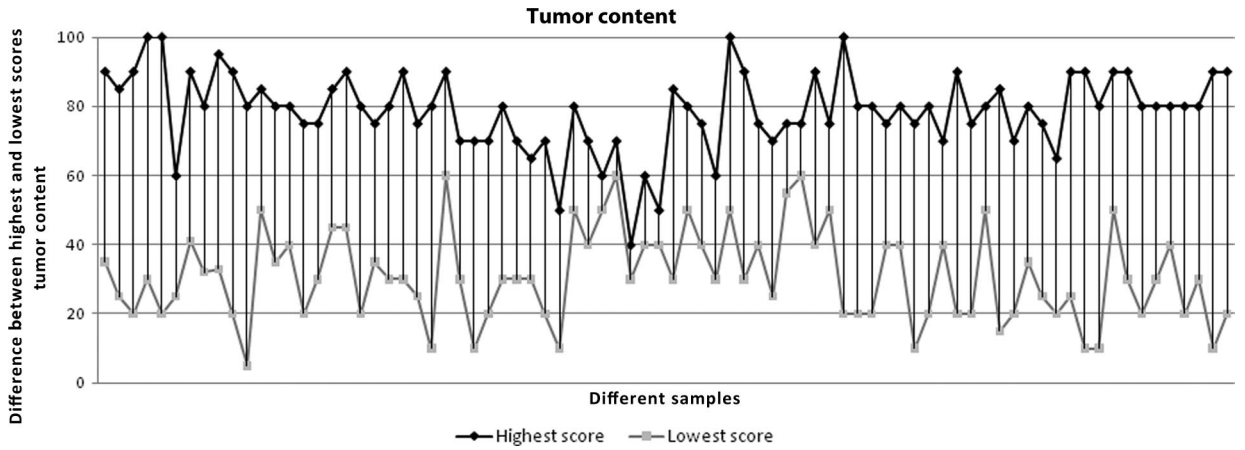

Figure 2. Results of tumor percentages in the different samples of the KRAS external quality assessment scheme.

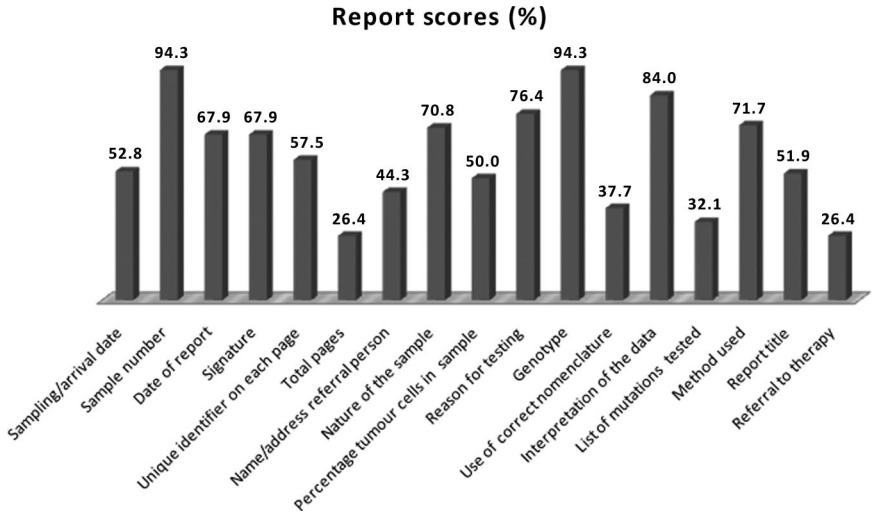

Figure 3. Scores of different items of the reports of the KRAS external quality assessment scheme, according to the ISO 15189: 2007 standard and Gulley et al. (2007) [14]. $n = 53$ reports analyzed.

ing the TheraScreen® DxS kit. Those that detected the c.34G>C (p.G12R) mutation were using in-house-developed allele-specific PCR or pyrosequencing analyses.

Types of Errors Encountered

We examined the most frequent mistakes made in *KRAS* testing in this EQA scheme in closer detail (Table 3). We observed five different errors: false-positive results (mutation instead of wild type), false-negative results (wild type instead of mutation), wrong mutation, isolation failure (DNA purification failed: no *KRAS* mutation detection was performed), and technical failure (*KRAS* mutation detection failed). Although the implications for patients differ, all mistakes were considered equally significant and were scored the same way. In total, 70% of the labs correctly identified all mutations. Of the 30% of laboratories that made at least one error, 22% made genotyping errors; the other 8% reported only technical failures. The majority of the errors were false-positive or false-negative results. In total, six false-negative and three false-positive results were reported (30% of the errors). For one sample, a different mutation was detected, and for five samples two mutations were detected, of which only one was correct. In total, eight technical or isolation failures were reported. Laboratories received enough sample material for *KRAS* mutation analysis, but it was not always possible to repeat the test with the leftover material/DNA in cases of technical errors. In clinical settings, in this case new slides would be requested to repeat the test. However, in this EQA scheme, we could not send an extra sample set to these laboratories because of limited patient sample material.

Reporting of Results

In total, 53 of 59 laboratories submitted reports for the three first cases. The mean score of the reports was 10 points (of a maximum of 17). Items that were usually included were

the sample number, genotype, and interpretation (conclusion, discussion) of the data. Correct numbering of pages, including the total number of pages, was absent in 75% of the reports (Fig. 3). Other items that were often not included in reports were the list of mutations tested, a name and/or address of the person the report was referred to, and a referral to anti-EGFR therapy if a positive result was found. Another observation was that 70% of the laboratories did not use correct nomenclature according to HGVS.

DISCUSSION

EGFR has been validated as a therapeutic target in colorectal cancer. However, these therapies are effective only in patients harboring a tumor with a wild-type *KRAS* gene [15, 16]. The appropriate selection of patients with colorectal cancer for treatment with anti-EGFR drugs is a major challenge. The purpose of the European *KRAS* EQA scheme is to provide optimal and reliable *KRAS* testing for patients throughout Europe. EQA schemes are designed to assess the accuracy and reliability of laboratory tests. The goal of this *KRAS* EQA scheme was to use quality assessment to enhance the performance of laboratories by assuring the quality of their *KRAS* mutation testing and by ensuring parity in test outcomes among participating laboratories, compared with a reference laboratory. A second goal was to use this scheme as a vehicle to develop a collaborative network of laboratories and thereby to encourage regional distribution of laboratory knowledge and expertise, to facilitate troubleshooting, and to support laboratories to set up *KRAS* mutation tests including the QA requirements.

An important outcome of this EQA scheme was the difference in mutations among the regional schemes. The regional scheme organizers were allowed to choose the mutations, resulting in variation among the different subschemes. To enhance equality among schemes in subsequent rounds, scheme organizers will be asked to send samples with the same mutations to participants. Our results showed that only 41 laboratories reported all 10 genotypes correctly when compared with the correct result, 14 laboratories made one genotype mistake, and four laboratories made two mistakes. Genotyping mistakes can be a result of several reasons. A very important issue is the starting material. One important factor regarding the starting material that will have an impact on the quality of the assay is the type of fixative used. Some fixatives, such as decalcification and picric acid–containing solutions, do not allow molecular testing [17]. The regional laboratory of scheme A used nonbuffered formalin, leading to lower DNA quality. This might be the cause of the relatively high number of failures in that subscheme.

Another important issue in *KRAS* genotyping is the method used for testing. Two basic methods are predominately used for *KRAS* testing: dideoxysequencing and allele-specific PCR. Sequencing has long been the most widely used method to detect point mutations. The major disadvantage is that sequencing is not very sensitive [18], and in samples with a low tumor content, in particular, analysis might be difficult [19]. Allele-specific PCR is more sensitive but tests for only a subset of the most common mutations, whereas sequencing can detect all possible mutations. A number of new technologies have also been applied to *KRAS* testing, such as high-resolution melting analysis and pyrosequencing. The latter has the advantage of higher sensitivity than regular dideoxysequencing [20].

In accordance with a recent review, two CE-marked *KRAS* mutation test kits currently exist in Europe for diagnostic use: TheraScreen® DxS and *KRAS* LightMix® (TIB MolBiol, Berlin, Germany) [8]. The majority of laboratories (40.3%) use the TheraScreen® DxS test kit for *KRAS* mutation testing. This kit is considered to be the gold standard for *KRAS* testing; however, in this EQA scheme, several mistakes were made using this kit. In addition, the kit is designed to detect only one mutation in a sample, and therefore the mutation scoring ignores possible double mutations, interpreting it as crosstalk. The c.34_35delinsAT (p.G12L) mutation was thus diagnosed as c.34G>A or c.35G>T (depending on the assay). Currently, we do not have enough data to provide information on a possible association between the method used for testing and the number and kind of mistakes. Over time, as more data become available, this scheme will be able to provide more detailed information about how different methods perform. Another important observation in this *KRAS* EQA scheme was the very high variability among laboratories in the estimation of the percentage of tumor cells in H&E-stained paraffin sections. This result can be explained by the choice of the region on which the analysis was done and by observer variability. We are presently analyzing the reason for this discrepancy using digitized slides. Based on this analysis, we will develop an e-tool for comparison of actual cases with example images. This work is in progress.

As with EQA schemes in general, there is concern that reported results may not reflect actual practice, given the possibility that EQA samples may be treated differently from routine clinical samples when tested. By requiring the return of results within 10 working days, we tried to minimize this influence. Whether or not it occurs, our data indicate that not all laboratories are capable of producing results of a high standard in *KRAS* testing. If this EQA scheme reflects *KRAS* testing on a routine basis, at least one in 10

samples is wrongly genotyped in >30% of laboratories. Incomplete or inaccurate exams lead to incorrect diagnoses and can have important consequences for a patient. There is an important difference among errors resulting in no diagnosis (technical failure) and requesting a new sample (which results in a time delay for the patient), errors resulting in no difference in diagnosis (different mutation), and errors resulting in a wrong diagnosis for the patient (false-positive or false-negative results). These differences were not taken into account in the scoring of the EQA scheme. In the light of quality control, we considered all mistakes equally important. For a laboratory, however, it might be useful to reflect on the possible consequences of a mistake in diagnosis.

An important aspect of diagnostic testing is reporting the results. There is a section on reporting of results in the ISO 15189 standard and a guideline document regarding reporting of molecular results in general [14]. The basic requirements of a report are information on the laboratory, patient, and sample identifiers; results; methodology; and interpretation. The general quality of the reports received in the context of this EQA scheme, judged by the requirements mentioned above, was very poor. Reporting is an essential step in diagnostic testing, and a clear and complete report is a very important issue. Good diagnostic tests are useless if the information is not transferred correctly to the physician requesting the test and, ultimately, the patient. Missing elements in a report could provide insufficient or even wrong information. For example, if no mutations are found, it is very useful to know which mutations were tested for and which were not, and a list of mutations that was tested for should be mentioned.

The results of this *KRAS* EQA scheme showed that several laboratories were making mistakes, such as misgenotyping of samples, and the overall quality of *KRAS* testing could be improved. EQA schemes are designed to assess the accuracy and reliability of laboratory tests. They identify, and subsequently minimize, discrepancies among results [21, 22]. Regular participation in EQA schemes facilitates improvement in testing processes when necessary and leads to standardization of test outcomes across participating laboratories. Further development of the *KRAS* EQA scheme aims to provide a baseline picture of the accuracy and reliability of the analysis of the *KRAS* test (e.g., number of mistakes, kind of errors), to identify areas of particular difficulty in testing procedures (e.g., detection of double mutations), and to provide a mechanism for improvement (e.g., more sensitive methods) for the participating laboratories.

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Final approval of manuscript: Elisabeth Dequeker, Marjolijn J.L. Ligtenberg, Sabine Tejpar, Gert de Hertogh, Karin De Stricker, Anders Edsjö, Vassilis Gorgoulis, Athanassios Kotsinas, Gerald Höfler, Andreas Jung, Pierre Laurent-Puig, Fernando López-Ríos, Etienne Rouleau, Tine Plato Hansen, Peter Vandenberghe, Johan J.M. van Krieken

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